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# Reproductive Behaviors of Male and Female Blackspotted Topminnows, *Fundulus olivaceus*

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The University of Southern Mississippi

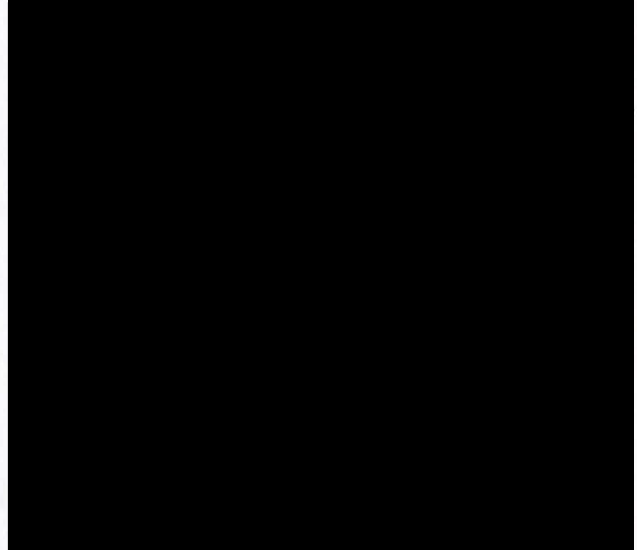
REPRODUCTIVE BEHAVIORS OF MALE AND FEMALE BLACKSPOTTED  
TOPMINNOWS, *FUNDULUS OLIVACEUS*

by

Melissa Ann Gutierrez

A Thesis  
Submitted to the Graduate School  
of The University of Southern Mississippi  
in Partial Fulfillment of the Requirements  
for the Degree of Master of Science

Approved:



Dean of the Graduate School

August 2010

## ABSTRACT

### REPRODUCTIVE BEHAVIORS OF MALE AND FEMALE BLACKSPOTTED TOPMINNOWS, *FUNDULUS OLIVACEUS*

by Melissa Ann Gutierrez

August 2010

The aim of the study was to characterize the reproductive behaviors of male and female blackspotted topminnows in the Pascagoula Drainage. I focused on phenotypic traits, size and number of dorsolateral spots, in males that possibly could cue a female to choose one male more frequently than other males present in the spawning group. With the use of microsatellite markers, I was able to determine parentage in trials where a single female was allowed to choose among phenotypically different males. I found that in all trials one male mated with the female(s) present. I also found that in all cases the dominant male exhibited the secondary characteristic of large body size and, in most cases, a high number of dorsolateral spots compared to the other males. In the breeding tank, the dominant male did not defend a particular spawning territory but mated freely among the available spawning substrates. Concluding a nonrandom mating pattern was observed when females were presented with several males. These results suggest that females have a preference for large males with a high number of dorsolateral spots, and males that possess secondary sex characteristics of large size and higher frequency of dorsolateral spots mate more frequently than males that do not.



## ACKNOWLEDGMENTS

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## CHAPTER I

## INTRODUCTION

In a promiscuous mating system, both males and females are able to mate freely among potential partners (Draud and Itzkowitz 2004; Baker et al. 2001). In such a system, genetic diversity and male and female mating success is dependent on the number of fertilization encounters and is not thought to be linked to whether the mate present has a preferable physical attribute (Draud and Itzkowitz 2004). Although selection for a mate is thought to decrease in promiscuous systems, females in promiscuous mating systems seem to exhibit similar preferences to females in polygynous systems (Dosen and Montgomerie 2004). Females, whose gamete production has a greater energetic cost compared to males, appear to select males that are the largest, most noticeable males that guard the preferred mating grounds (Jacobs et al. 2009; Kodric-Brown 1977). Such traits are visual cues to the female that the dominant male is preferred over other males. Secondary sexual characteristics, traits that distinguish sexes from one another, are also sometimes a positive indicator of male quality (Jacobs et al. 2009). Such physical attributes are correlated with a male's ability to forage successfully, testes size, and overall health (Jacobs et al. 2009; Baker et al. 2001). By choosing a male with preferred secondary sexual characteristics, the female may avoid disease transfer and the possibility of hybridization. Indirectly, the female may also increase the fitness of her male offspring in that they may inherit the desirable secondary sexual characteristics (Dosen and Montgomerie 2004).

Within the family Fundulidae, a range of coloration, habitat use, territory establishment, and reproductive behaviors have been described (Ross 2001). *Fundulus*



*notatus*, *F. olivaceus*, and *F. euryzonus*, which make up the *Fundulus notatus* complex, are similar in appearance to one another but are distinguishable from the other *Fundulus* species by the dark lateral band that extends from the anterior to posterior end of the fish (Ross 2001; Blanchard 1996; Thomerson, 1966). Like other Fundulids, the three identified species of *Fundulus notatus* complex are iteroparous mating multiple times throughout a reproductive season (Carranza and Winn 1954). Within a species, males and females are described as promiscuous and mate freely among the males and females within the species (Carranza and Winn 1954).

Although the three species are similar in appearance, phenotypic differences exist among the species. *Fundulus notatus*, the blackstripe topminnow, is the smallest species of the three and inhabits areas from Michigan to areas surrounding the Gulf of Mexico (Ross 2001). *F. notatus*, when compared with the other species, has relatively few spots located on the dorsal area of the body (Blanchard 1996; Braasch and Smith 1965). On the other hand, *F. euryzonus*, the broadstripe topminnow, with the smallest range endemic to the Lake Pontchartrain Drainage, is recognized by the dark lateral band that covers more than one third of its body depth (Ross 2001; Blanchard 1996). Lastly, *F. olivaceus*, the blackspotted topminnow, found in tributaries of the Mississippi River and coastal drainages as far east as the panhandle of Florida, is distinguishable from the other two species by the extensive number of spots found above the lateral band (Ross 2001; Braasch and Smith 1965).

Sexual dimorphism between males and females of the *Fundulus notatus* complex is recognized by several physical characteristics (Carranza and Winn 1954). The male topminnows have extended, pointy dorsal and anal fin; whereas, the female topminnows



have short, rounded dorsal and anal fins (Ross 2001; Carranza and Winn 1954). Secondly, coloration of dorsal and anal fins in males are typically yellow, whereas, females lack color on fins (Carranza and Winn 1954). For *F. olivaceus*, the presence of spots on the dorsolateral surface is a secondary sexual characteristic. From unpublished data collected by Jake Schaefer (2010), the number of dorsolateral spots increases with male overall size; whereas, dorsolateral spot number in females is not correlated to size and remains relatively constant despite body size (Figures 1 and 2). In males, spot number and gonadosomatic index, or GSI, exhibit a positive linear relationship (Figure 3), indicating that spot number may serve as a visual indicator of male reproductive potential, which could also act as an indicator of male dominance and sperm quality (Briskie 1993). In females, spot number is not correlated to gonadosomatic index and remains relatively constant as body size increases (Figure 4).

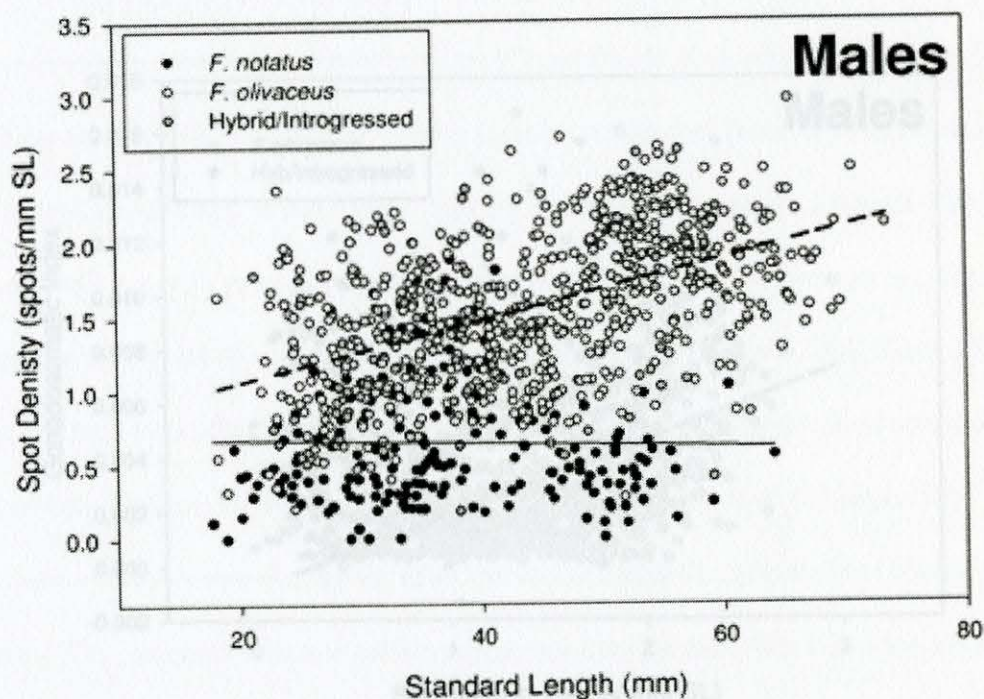


Figure 1. Spot density in relation to standard length of male *Fundulus notatus*, *F. olivaceus*, and hybrid/introgressed males.



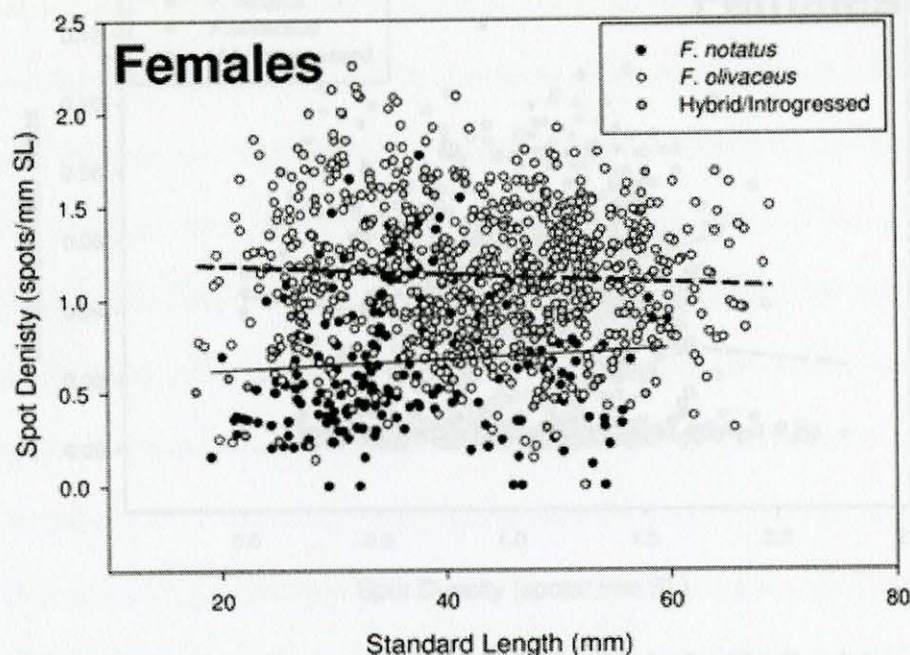


Figure 2. Spot density in relation to standard length of female *Fundulus notatus*, *F. olivaceus*, and hybrid/introgressed females.

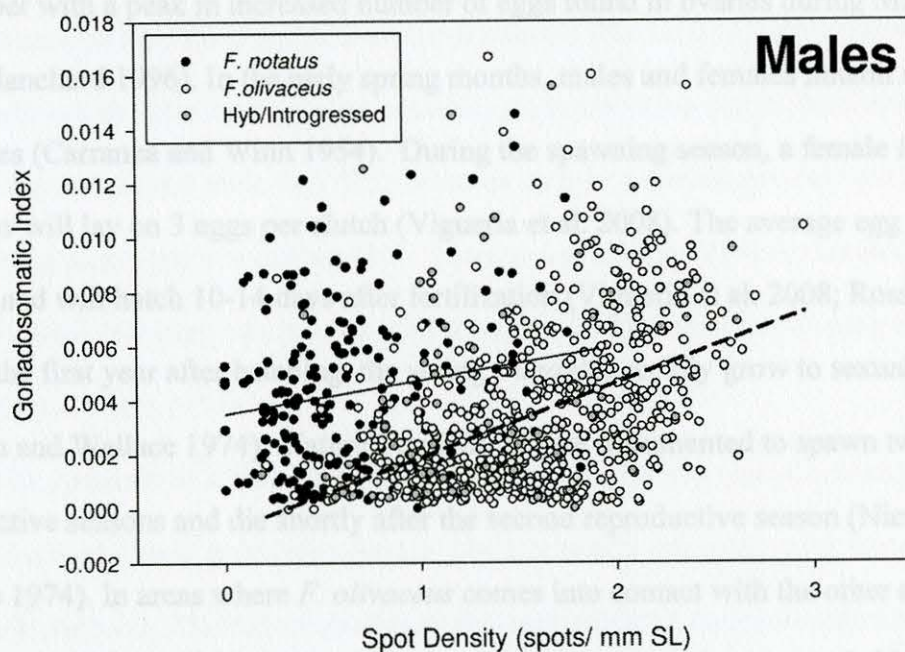


Figure 3. Gonadosomatic index in relation to spot density in male *Fundulus notatus*, *F. olivaceus*, and hybrid/introgressed males.

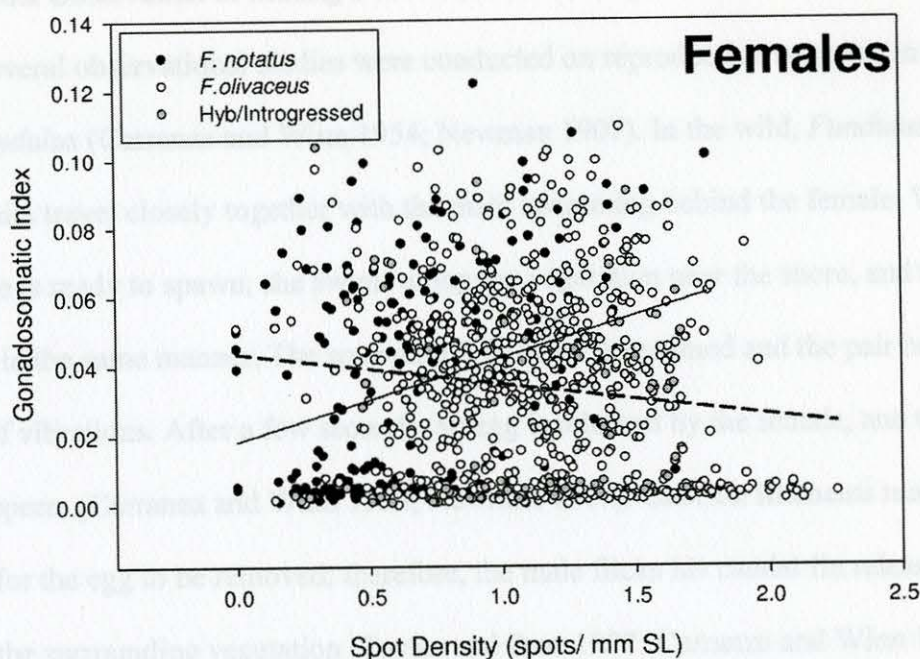


Figure 4. Gonadosomatic index in relation to spot density in female *Fundulus notatus*, *F. olivaceus*, and hybrid/introgressed females.

*Fundulus olivaceus* reproductive seasons begins during late spring and ends in September with a peak in increased number of eggs found in ovaries during May (Ross 2001; Blanchard 1996). In the early spring months, males and females inhabit shallow shorelines (Carranza and Winn 1954). During the spawning season, a female *Fundulus olivaceus* will lay on 3 eggs per clutch (Vigueria et al. 2008). The average egg size is 1.8-1.9 mm and will hatch 10-14 days after fertilization (Vigueria et al. 2008; Ross 2001). During the first year after hatching, the young *Fundulus* rapidly grow to sexual maturity (Nieman and Wallace 1974). Mature *Fundulus* sp. are documented to spawn two reproductive seasons and die shortly after the second reproductive season (Nieman and Wallace 1974). In areas where *F. olivaceus* comes into contact with the other species (contact zone) of the *Fundulus notatus* complex, hybrids have been recorded in such areas (Duvernell et al. 2007).



## Visual Observation of Mating Behavior and Territory in the Genus *Fundulus*

Several observational studies were conducted on reproductive behavior in the genus *Fundulus* (Carranza and Winn 1954; Newman 1907). In the wild, *Fundulus notatus* mating pairs travel closely together with the male swimming behind the female. When the female is ready to spawn, she swims along the vegetation near the shore, and the male proceeds in the same manner. The spawning S position is assumed and the pair beginning a series of vibrations. After a few seconds, an egg is released by the female, and the male releases sperm (Carranza and Winn 1954; Newman 1907). Chorion filaments make it difficult for the egg to be removed; therefore, the male flicks his caudal fin releasing the egg into the surrounding vegetation (Taylor and Burr 1997; Carranza and Winn 1954; Richardson 1939). The behavior has also been observed in *Fundulus heteroclitus*, *Fundulus diaphanus*, and *Fundulus dispar* in which males actively pursue females (Taylor and Burr 1997; Richardson 1939; Newman 1907). When the pair is ready to mate, the male pushes the females towards vegetation (Richardson 1939). Newman (1907) believes such behavior guarantees an egg's fertilization due to the close proximity between the female and male fish. This behavior may be repeated several times by the same mating pair (Taylor and Burr 1997; Carranza and Winn 1954; Richardson 1939). A female *Fundulus notatus* was documented releasing 30 eggs in one spawning event (Carranza and Winn 1954).

The presence of a male does not always secure a mating pair. Carranza and Winn (1954) observed a male pursuing a female who eventually released viable eggs without the male's company. The unfertilized eggs fell onto substrate and were eaten by the female (Carranza and Winn 1954). The same behavior is observed if no male is present.



The female topminnow performs the vibrating technique to release ripe eggs, and as the eggs fall to substrate, the female consumes the unfertilized eggs (Carranza and Winn 1954). Similar behavior has been observed in wild and laboratory mating of *Fundulus heteroclitus* and *Fundulus notatus* (Carranza and Winn 1954; Newman 1907).

Observational studies suggest the largest male *Fundulus notatus* in a group is an aggressive dominant that will chase smaller males from females (Ross 2001; Carranza and Winn 1954). The largest male will also spawn with all of the reproductive females in a confined tank with multiple males and females present. Rarely does a smaller male mate with any female present. If mating between a smaller male and female does occur, it is on the opposite side of the tank while the dominant male is courting another female (Carranza and Winn 1954).

In the wild, Fundulidae are found in areas with abundant vegetation (Blanchard 1996). Description of territorial behaviors in the wild has been described as weak to none (Carranza and Winn 1954). Within these weak territories, male size determines the social status of the male and size of the territory (Carranza and Winn 1954).

#### Purpose of Research

The purpose of this research is to focus on mate selection and preference for a secondary trait that may also serve as a reproductive isolation mechanism in *Fundulus olivaceus*. Through paternity analysis of offspring, the number of males actually mating with the available females will be determined. I predict that the mating male will most often be the largest and most heavily spotted individual.

Whether male *F. olivaceus* mate in one or multiple spawning mops will also be addressed. *Fundulus olivaceus* are obligatory plant spawners found near emergent

vegetation (Carranza and Winn 1954). Within the vegetation, males are found in territories related to male body size (Carranza and Winn 1954). Multiple pairs may be found in close proximity, but the largest male tends to occupy the center and largest territory (Carranza and Winn 1954).

#### Research Hypotheses

I hypothesize that: 1) within a group of several breeding males, a female will select for the secondary characteristics of large body size and high density of dorsolateral spots, 2) male *F. olivaceus* will guard a limited territory and mate in that area exclusive of other areas, and 3) one males will remain dominant over time and sire all offspring resulting from a given trial. The null hypotheses are that mating will be random, each male will sire an equal portion of offspring, and eggs will be evenly dispersed among spawning media.

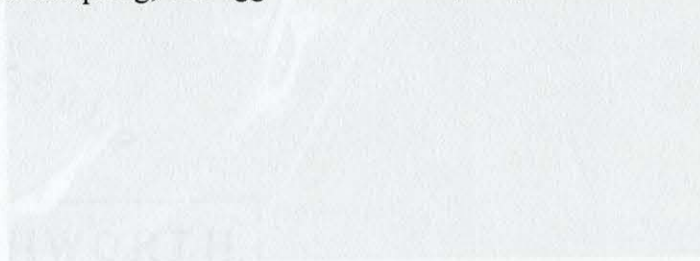


Figure 3. Black Creek collection site for *Fundulus olivaceus*. Image from GoogleEarth.

Adult specimens of *F. olivaceus* were collected using mesh seines and dippers in slow to moderate flowing water (Holcroft 2004; Rees 2001; Atmar and Stewart 1972; Thompson and Woolridge 1970; B. Zisch and Smith 1965). Fish removed from the environment were placed into a large ice chest filled with the current environmental water and an aerated pump. The fish were transported back to the USM wetlab facility located in Walker Science Building and acclimated to the present holding tank. All handling and animal care procedures were approved by the local IACUC committee.



## CHAPTER II

## MATERIALS AND METHODS

Administrative Scientific Collecting Permit from Mississippi Number 0605091 was received from the Museum of Natural Science to collect *F. olivaceus* from June 2009 to June 2010. Fish were collected from two sites near Hattiesburg in the Pascagoula Drainage: Black Creek and Okatoma Creek (Figure 5).



Figure 5. Black Creek collection site for *Fundulus olivaceus*. Image from GoogleEarth.

Adult specimens of *F. olivaceus* were collected using mesh seines and dipnets in slow to moderate flowing water (Holcroft 2004; Ross 2001; Atmar and Stewart 1972; Thomerson and Woolridge 1970; Braasch and Smith 1965). Fish removed from the environment were placed into a large ice chest filled with the current environmental water and an aerated pump. The fish were transported back to the USM wetlab facility located in Walker Science Building and acclimated to the present holding tank. All handling and animal care procedures were approved by the local IACUC committee.



Mesocosm experiments on the reproductive behaviors of *Fundulus olivaceus* took place indoors in the Walker Science Building. Fish were kept in glass and/or fiberglass holding tanks located in the Walker Science Building. Photoperiod and temperature were regulated to mimic environmental conditions experienced in the wild during the reproductive season. The fish were fed daily with alterations between frozen brine shrimp and freeze dried blood worms.

At the beginning of the experiment, fish were separated and assigned to individual holding tanks. Each fish was anesthetized using MS-222. A fish was placed on a white board, assigned a permanent ID, and photographed for size and spot counting. Next, a tissue sample from one of the pectoral fin was clipped and stored in a SED solution for DNA extraction. The fish was returned to the isolated holding tank until experiments began.

DNA was extracted from the fin clips of males using a DNeasy Blood and Tissue kit from QIAGEN and then stored at -20° C. Several microsatellite loci developed for *F. heteroclitus* were found to be variable in *F. olivaceus* (Adams et al. 2005). I used one polymorphic locus (FhATG-B103) to identify males with unique genotypes for use in the mating trials. These individuals were then sorted based on phenotypic characteristics (body size and number of spots) prior to trials.

Polymerase chain reaction (PCR) amplifications were conducted in a total volume of 12.5  $\mu$ L using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 1.5–2.0 mM  $MgCl_2$ , 200  $\mu$ M dNTPs, 0.3  $\mu$ M of the M13 tailed forward primer (Boutin-Ganache et al., 2001), 0.3  $\mu$ M of the reverse primer, 0.1  $\mu$ M of the M13 labeled primer (LI-COR), 0.1875 units of *Taq* polymerase (New England Biolabs), 20–100 ng of template DNA



and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 94 C for 2 min followed by 35 cycles of 30 sec at 94 C, 1 min at 56–60 C and 1 min at 72 C. A final elongation step of 10 min at 72 C ended the cycle. I visualized the microsatellite alleles using a LI-COR 4300 DNA Analysis system and scored them using Gene Image IR v. 3.55 (LI-COR) (Figure 6).

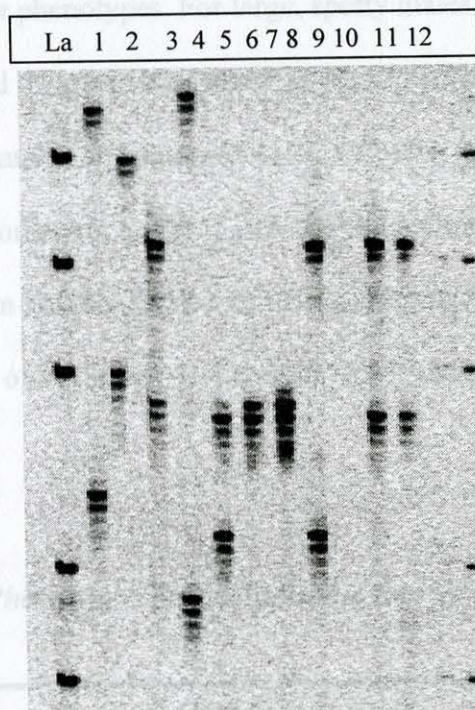


Figure 6. Li-Cor 4300 DNA Analyzer Gel Electrophoresis. Photograph was taken August 14, 2009 depicting the genotypes of four *F. olivaceus* males, one female, and six offspring in Trial 3D using the primer set B103. La is 350 bp ladder lane. 1 is small, not spotty male; 2 is large, not spotty male; 3 is large, spotty male; 4 is small, spotty male. 5 is the female used in the trial. 6-12 is the larval offspring collected and used to identify the paternal line.

From genotype data, *F. olivaceus* males were separated from other males with identical homozygous or heterozygous genotypes. Individuals were then placed within a spawning group based on phenotypic characteristics (body size and number of spots) prior to trials. The mean body size ( $57.7 \text{ spots} \pm 21.9 \text{ SD}$ ) and mean spot count (Mean:



5.47 cm  $\pm$  0.78 SD) represents all males (n=52) used for mating experiments. Males larger than the mean body size were classified as "large" while those smaller than the mean were classified as "small." Similarly, males were considered spotty or not spotty if the number of spots on the dorsal lateral region was above or below the group means (Table 1). The mean and standard error for the size and spot density was determined for the males placed into the four phenotypes. For large, spotty males, the mean number of spots was  $83.1 \pm 4.41$  SEM, and the mean size was  $6.27 \pm 0.12$  SEM (Figure 7). For large, not spotty males, the mean number of spots was  $45 \pm 2.44$  SEM, and the mean size was  $5.84 \pm 0.10$  SEM (Figure 7). For small, spotty males, the mean number of spots was  $71.25 \pm 5.36$  SEM, and the mean size was  $5.21 \pm 0.11$  SEM (Figure 7). For small, not spotty males, the mean number of spots was  $41.9 \pm 1.99$  SEM, and the mean size was  $4.73 \pm 0.08$  SEM (Figure 7).

Table 1

*Determining a Male's Phenotype within a Spawning Group*

Phenotypes	Mean	> Mean	< Mean	Range
1. Spots	57.7 spots $\pm$ 21.9	>57.7 Spotty	<57.7 Not Spotty	25-109
2. Size	5.47 cm $\pm$ 0.78	>5.47 cm Large	<5.47cm Small	4.14-7.23 cm

Note: L S: Large and Spotty were males above the mean for size and spot density; L NS: Large and Not Spotty were males that were above the mean for size and below the mean for spot density; S S: Small and Spotty were males were males below the mean for size and spot density; S NS: Small and Not Spotty were males that were below the mean for size and above the mean for spot density.

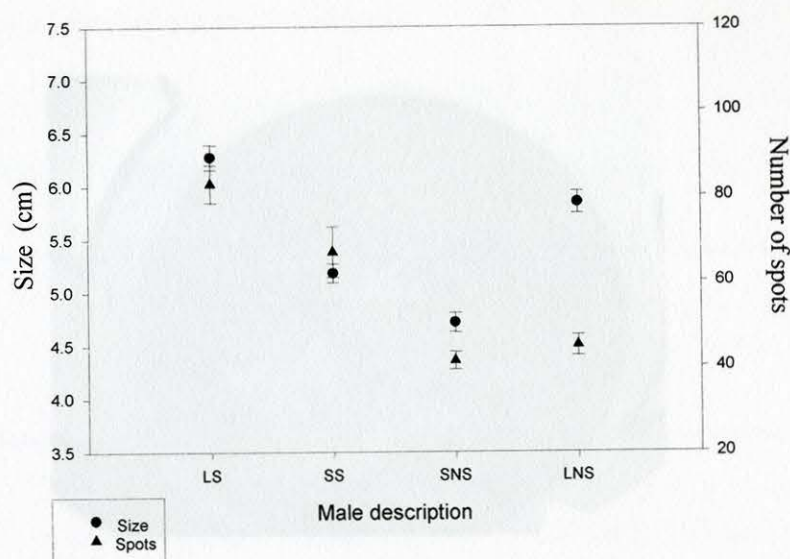


Figure 7. The mean and standard error of the following phenotypes: Large and Spotty, Small and Spotty, Small and Not Spotty, and Large and Not Spotty.

#### Mating Trials with One Female *Fundulus olivaceus*

Mating trials took place in 6 separate tanks, each with four spawning substrates, four males (a spawning group), and one female (Figure 8). Spawning substrates were made of Red Heart acrylic/polyester yarn in dark sage. The mops were composed of 80 to 100 individual strands of yarn. The mops were used to mimic natural vegetation and provided a place for the female to lay and conceal her eggs. Four mops were used to provide each male a potential territory.

Males in spawning groups 1, 2, and 3 were selected so all four phenotypes: 1) large body with high number of spots (LS) 2) large body with low number of spots (LNS) 3) small body with high spot count and (SS) 4) small body with low number of spots (SNS), were included within the spawning tank. Trials ran until four clutches (a clutch is defined as eggs deposited in one 24 hour period) were collected from each group. For



each clutch, the number of eggs and the spawning substrate (mop) they were found were recorded.



Figure 8. Tank set up included four spawning mops (one per male *F. olivaceus*).

Eggs were incubated in plastic storage containers and treated daily with fungicide as described in Vigueira et al. (2008). The eggs were incubated for approximately two weeks, until hatching, to increase the DNA extraction yields to enhance the success of subsequent genotyping. Late term eggs or newly hatched larvae were stored in SED solution (Seutin et al. 1991).

Spawning groups 4, 5, and 6, differed from the first three trials only by the random selection of males so that there was not always one of each phenotype in each spawning group (Table 3).

#### Repeated Use of the Same Spawning Group

Spawning groups 1, 2, and 3 were used for multiple trials with different females. To ensure that the phenotype assigned to the male remained the same in between trials, the males were photographed before and after each trial to determine the length and number of spots found on the dorsolateral region of the male. An example of the multiple spot counts for the males in a spawning group is found in Table 2. The paternity lines of

several mating trials were compared to determine whether or not the same male was selected by the female throughout the experiment.

Table 2

*The Mean and Standard Deviations of Spots Counts for Males in Spawning Group 1*

Phenotypes Dates Recorded	1 <sup>st</sup> 2-24-09	2 <sup>nd</sup> 5-18-09	3 <sup>rd</sup> 6-30-09	4 <sup>th</sup> 7-30-09	5 <sup>th</sup> 8-13-09	Mean $\pm$ SD
1. Spotty Large	67 S 7.23cm	70 S 7.5 cm	71 S -	68 S 7.97 cm	71 S 7.99 cm	69.4 $\pm$ 1.82
2. Spotty Small	69 S 5.45cm	67 S 6.0 cm	68 S -	69 S 6.82 cm	66 S 6.83 cm	67.8 $\pm$ 1.30
3. Not Spotty Small	39 S 4.93cm	44 S 5.53 cm	41 S -	39 S 6.17 cm	46 S 6.29 cm	41.8 $\pm$ 3.11
4. Not Spotty Large	54 S 5.98cm	56 S 6.4 cm	53 S -	52 S 7.02 cm	54 S 7.02 cm	52.6 $\pm$ 1.67

Note: (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup> trials). Each male was assigned to one of the four categories: Large Spotty; Large Not Spotty; Small Spotty; Small Not Spotty.)

### Mating Trials with Two Females

A second series of trials, 7-13, was conducted that differed from the previous by the presence of two females and the use of two available spawning substrates. In the following spawning groups, 7-13, a new set of males were randomly assigned to a spawning group. After males were assigned to a spawning group, the phenotype of the males were described. Trial length, extraction of eggs, incubation and eventual genotyping protocols were the same for these trials. A complete list of the male phenotypes in the 13 spawning groups is provided in Table 3.



Table 3

*Description of Males within a Spawning Group*

Male Spawning Group	Phenotypes of Males Within the Group	Number of Trials and Number of Eggs	Number of Females
1	LS, LNS, SS, SNS	5:156	1
2	LS, LNS, SS, SNS	3:84	1
3	LS, LNS, SS, SNS	5:150	1
4	LS, LNS, SNS, SNS	1:36	1
5	LS, LNS, LNS, SNS	1:17	1
6	LS, LNS, LNS, SNS	1:23	1
7	LS, SS, SNS, SNS	1:29	2
8	LS, LS, SNS, SNS	1:15	2
9	LS, LS, LNS, SNS	1:20	2
10	LS, LS, LNS, SNS	1:9	2
11	LNS, LNS, SS, SS	1:28	2
12	LS, SNS, SNS, SNS	1:32	2
13	LNS, LNS, SS, SS	1:54	2

Note: Each male was assigned to one of the four categories: Large and Spotty; Large and Not Spotty; Small and Spotty; Small and Not Spotty, in a spawning group.

## Statistical Analysis

All descriptive statistics, Chi Square test, and one factor, one way analysis of variance were done using JMP 8 software. All data were tested for normality and transformed appropriately if necessary.

## CHAPTER III

### RESULTS

Trials ran from March 2009 to November of 2009 and yielded 653 eggs from 35 mate-choice trials. Of the 35 trials, 26 consisted of one female and four males and 9 consisted of two females and four males. In 10 trials, mating was not observed. In trials in which no mating was observed, females were left for 3 to 4 weeks before being replaced. The minimum trial length was four days. Two trials were terminated due to male mortality and data collected were not used in any of the data analyses. Hereafter, data are only presented for the 23 trials that produced viable eggs.

#### Egg Production

For all trials, the daily mean egg production for female *F. olivaceus* was  $2.79 \pm 2.17$  eggs per day. Of the 653 eggs collected, 481 successfully hatched (73.66% hatching success). The mean number of days for an egg to hatch was  $17.44 \pm 3.84$  days.

#### Genotypic Analysis

Of the 481 larval fish that were collected from the mate choice trials, 184 larval fish were genotyped and paternity was successfully determined for all.

#### Mate Choice Experiments

From the genotyping analysis, only one male was found to mate with the available female (Table 4). In trials in which the same males were used repeatedly, the same male remained dominant over multiple trials and sired all offspring. In spawning group 1, the large and not spotty male, was the selected male throughout the reproductive trials and sired all of the genotyped offspring (Table 4). In spawning group 2 and 3, the large and



spotty males were the dominant males, fertilizing all eggs in their respective trials (Table 4).

Table 4

*Parentage Results for Spawning Groups 1-3*

Male Group	# of Females	Total # Eggs Collected	Number of Trials					% of Offspring Genotyped
			Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	
1	1	156	LNS(12)	LNS(5)	LNS(7)	LNS(8)	LNS(14)	30.1%
2	1	84	LS(6)	LS(7)	LS(10)	NA	NA	27.4%
3	1	150	LS(8)	LS(5)	LS(4)	LS(8)	LS(14)	19.3%

Note: In each group, one of the following types of males was included: L S: Large and Spotty; L NS: Large and Not Spotty; S S: Small and Spotty; S NS: Small and Not Spotty assigned to a specific tank. # of Females represents the number of females placed in the tank. # of Eggs Collected is the number of eggs found in all spawning media in the tank. Trials represent the number of times a group of males was used. For each group, at least one trial was successful. Within the trial, the male identified as the father of the offspring is identified by his phenotype. Underneath the males phenotype is the number of successful offspring that were genotyped. % of Offspring Genotyped is the percentage of all the genotyped eggs compared to the total number of eggs collected.

Of the 390 eggs, all eggs were fertilized by a large male ( $X^2 (1, N = 390) = 390, p < 0.001$ ) (Figure 9). 156 eggs were fertilized by a spotty male; while, 234 eggs were fertilized by a not spotty male in the tank ( $X^2 (1, N = 390) = 15.6, p < 0.001$ ) finding there is a significant difference between the number of eggs fertilized by a spotty male and not spotty male (Figure 10).

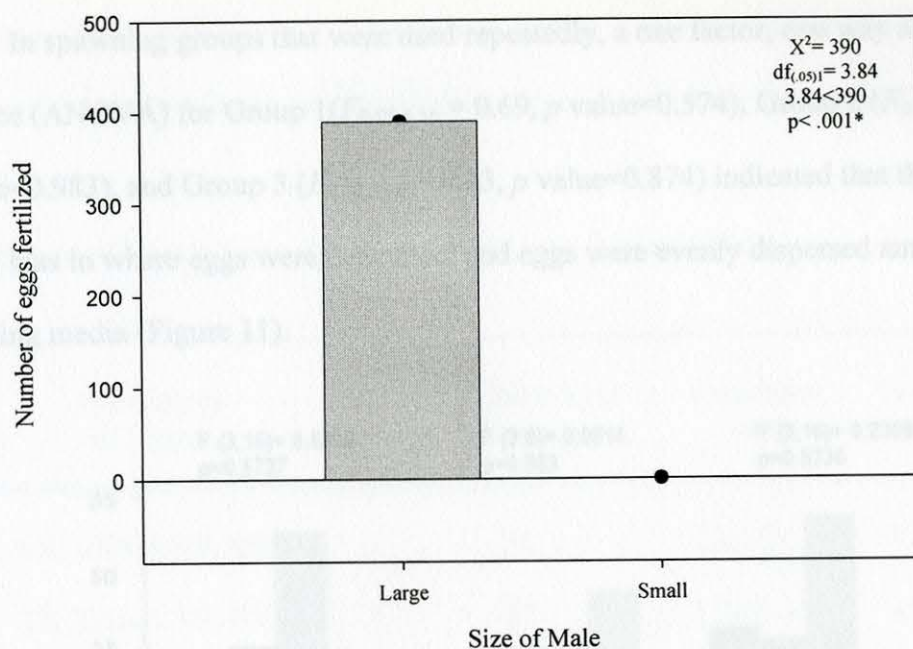


Figure 9. Number of eggs fertilized in relation to the size of the male that fertilized the eggs.

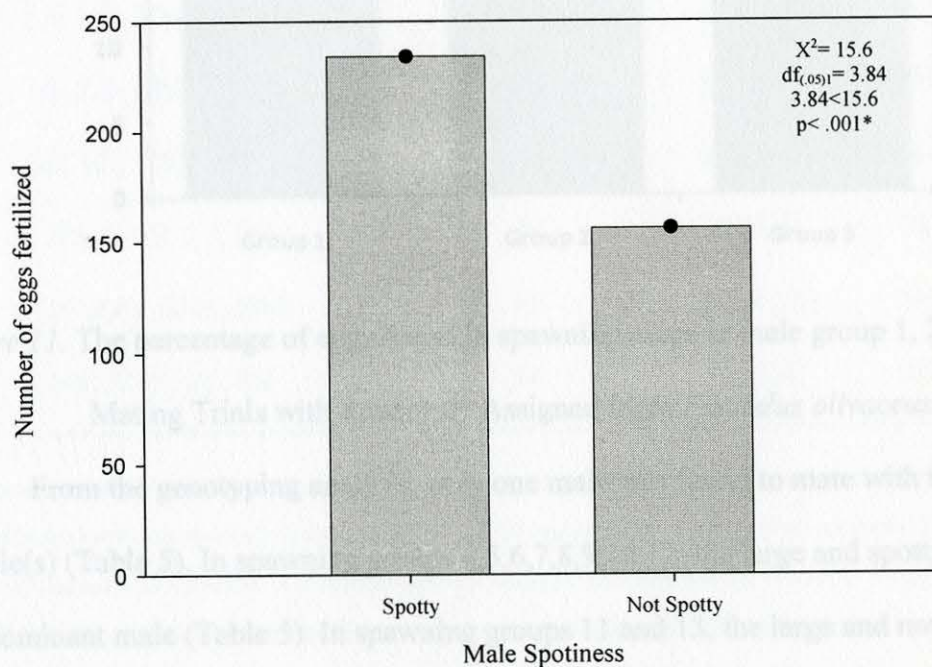


Figure 10. Number of eggs fertilized in relation to the spottiness of the male that fertilized the eggs.



## Territorial behavior

In spawning groups that were used repeatedly, a one factor, one way analysis of variance (ANOVA) for Group 1 ( $F_{0.05,3,16} = 0.69$ ,  $p$  value=0.574), Group 2 ( $F_{0.05,3,8} = 0.05$ ,  $p$  value=0.983), and Group 3 ( $F_{0.05,3,16} = 0.23$ ,  $p$  value=0.874) indicated that there was no spatial bias in where eggs were deposited, and eggs were evenly dispersed among spawning media (Figure 11).

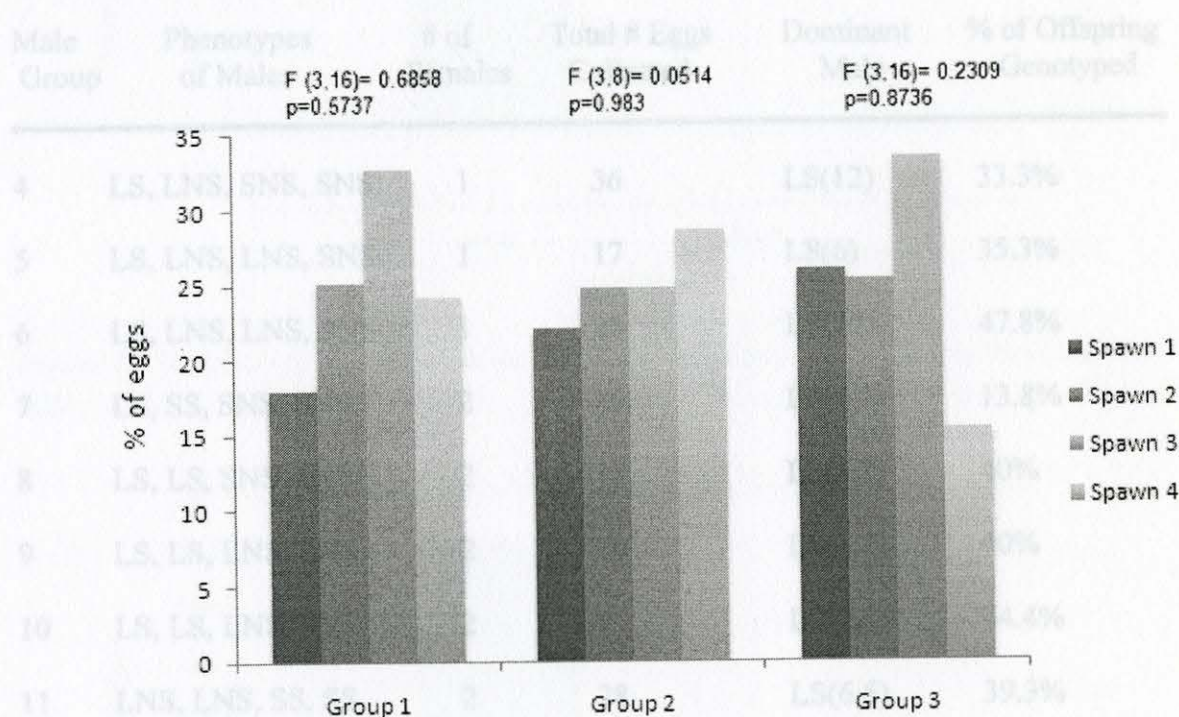


Figure 11. The percentage of eggs found in spawning mops in male group 1, 2, and 3.

Mating Trials with Randomly Assigned Male *Fundulus olivaceus*

From the genotyping analysis, only one male was found to mate with the available female(s) (Table 5). In spawning groups 4,5,6,7,8,9,10,12, the large and spotty male was the dominant male (Table 5). In spawning groups 11 and 13, the large and not spotty male was the dominant male (Table 5). Of the 263 eggs, all eggs were fertilized by a large male ( $\chi^2 (1, N = 263) = 263$ ,  $p < 0.001$ ) (Figure 12). As for dorsolateral spots, 181

eggs were fertilized by a spotty male; while, 82 eggs were fertilized by a not spotty male in the tank ( $X^2 (1, N = 263) = 37.3, p < 0.001$ ) finding there is a significant difference between the number of eggs fertilized by a spotty male and not spotty male (Figure 13).

Table 5

*Description of Mate Choice Trials with Randomly Assigned Males*

Male Group	Phenotypes of Males	# of Females	Total # Eggs Collected	Dominant Male	% of Offspring Genotyped
4	LS, LNS, SNS, SNS	1	36	LS(12)	33.3%
5	LS, LNS, LNS, SNS	1	17	LS(6)	35.3%
6	LS, LNS, LNS, SNS	1	23	LS(11)	47.8%
7	LS, SS, SNS, SNS	2	29	LS(2,2)	13.8%
8	LS, LS, SNS, SNS	2	15	LS(4,2)	40%
9	LS, LS, LNS, SNS	2	20	LS(1,7)	40%
10	LS, LS, LNS, SNS	2	9	LS(2,2)	44.4%
11	LNS, LNS, SS, SS	2	28	LS(6,5)	39.3%
12	LS, SNS, SNS, SNS	2	32	LS(1,10)	34.4%
13	LNS, LNS, SS, SS	2	54	LS(5,6)	20.4%

Note: Spawning group of males represents a group of males in one spawning tank. In each group, the following male phenotypes included in the spawning group were L S: Large and Spotty; L NS: Large and Not Spotty; S S: Small and Spotty; S NS: Small and Not Spotty assigned to a specific tank. # of Females represents the number of females placed in the tank. # of Eggs Collected is the number of eggs found in all spawning media in the tank. Male selected is the identified father of the offspring, and underneath the selected male is the number offspring. For trials with 2 females, the numbers in parenthesis are the number of successfully genotyped offspring. The first number represents the number of eggs released by female one. The second number represents the number of eggs released by female two.



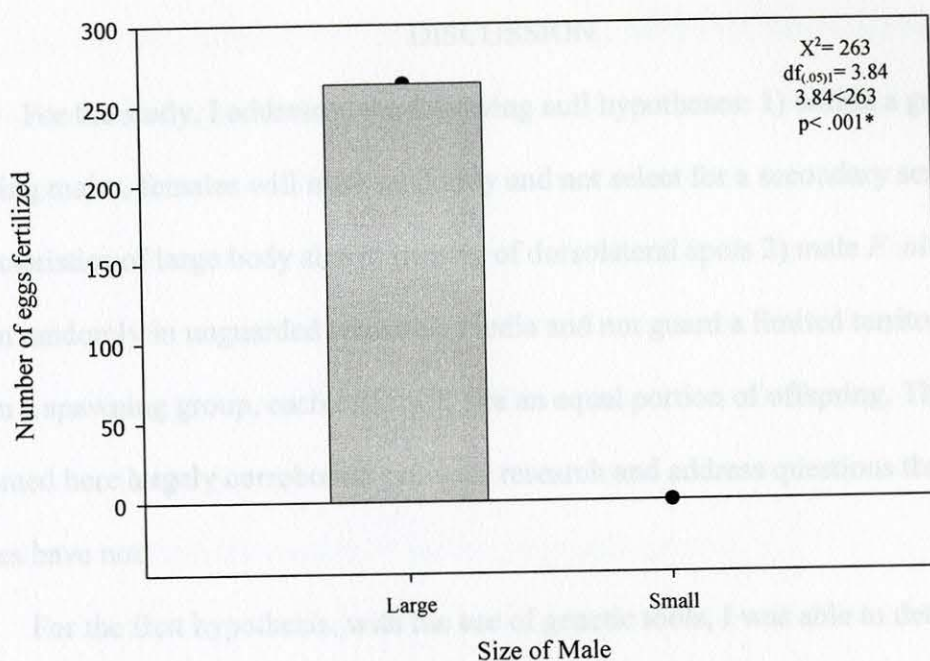


Figure 12. Number of eggs fertilized in relation to the size of the male that fertilized the eggs.

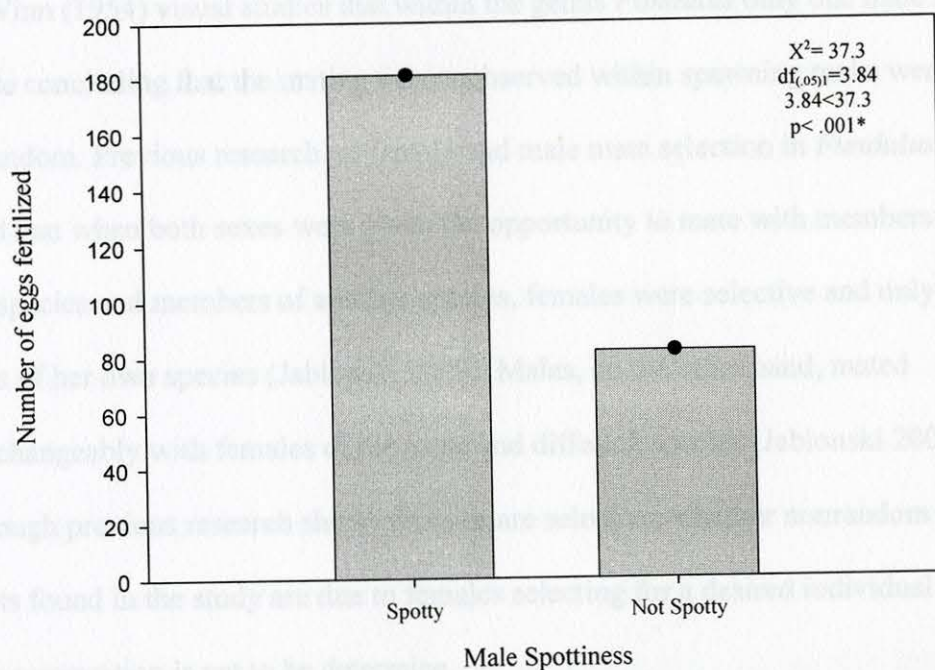


Figure 13. Number of eggs fertilized in relation to the spottiness of the male that fertilized the eggs.

## CHAPTER V

## DISCUSSION

For the study, I addressed the following null hypotheses: 1) within a group of breeding males, females will mate randomly and not select for a secondary sex characteristics of large body size or density of dorsolateral spots 2) male *F. olivaceus* will spawn randomly in unguarded spawning media and not guard a limited territory, and 3) within a spawning group, each male will sire an equal portion of offspring. The results presented here largely corroborate previous research and address questions that previous studies have not.

For the first hypothesis, with the use of genetic tools, I was able to determine paternity of *F. olivaceus* offspring within a spawning group and rejected the null hypothesis that random mating occurs in *F. olivaceus*. Such evidence supports Carranza and Winn (1954) visual studies that within the genus *Fundulus* only one male mates with female concluding that the mating events observed within spawning tanks were nonrandom. Previous research on female and male mate selection in *Fundulus olivaceus* found that when both sexes were given the opportunity to mate with members of their own species and members of another species, females were selective and only mated with males of her own species (Jablonski 2009). Males, on the other hand, mated interchangeably with females of the same and different species (Jablonski 2009). Although previous research shows females are selective, whether nonrandom mating events found in the study are due to females selecting for a desired individual or due to male competition is yet to be determine.



Within the spawning groups, the large male was always selected by the female suggesting that the secondary sex characteristic, large body size, maybe a positive indicator of male quality in *F. olivaceus* males. Like other mating systems, a high quality male within a mating system is often the largest male since size is related to a male's metabolism and overall health of the fish (Jacobs et al. 2009; Baker et al. 2001; Qvarnstrom & Forsgren 1998; Andersson 1994; Kodric-Brown 1977). Interestingly, in two trials with multiple large males, 7 and 10, the largest male in the spawning group was not always the male that mated with the females. In both trials, the large male that mated with the females was the smaller of the two large male present.

In the majority of the spawning groups, the male selected by the female was spotty. In three spawning group, the selected male was not spotty. In two of the three groups, Groups 8 and 10, a spotty male was not present in the mating trials. Without the presence of a spotty male, an inaccurate portrayal of female's selecting for either a spotty or not spotty male occurred due to the incapability for her to select for a spotty male.

Whether females are actively or passively selecting for spots is yet to be determined. Future studies should involve determining whether or not spots are an honest signal in *F. olivaceus*. In order for a signal to be honest, the signal must have a positive and a negative association with mate quality. Many dominant males possess exaggerated traits making the trait a positive indicator of mate quality. At the same time, the cost of the exaggerated trait decreases the survival of the male due to the conspicuous demeanor of the signal that makes the male at a high risk for predation (Veiga 1993). A study on a predator selection on spotty and not spotty males should be considered to accurately address the validity of spottiness as a mate quality indicator. Similar studies conducted on



the Trinidad guppy (Poeciliidae), *Poecilia reticulata*, found that the expression of spots differs for males depending on whether or not the area is of high or low predation (Stoner and Breden 1988) suggesting that spots found on the male increases predation risk and is an honest signal (Endler 1980).

For the second hypothesis, I failed to reject the null hypothesis concluding that males do not guard a particular spawning media and females disperse their eggs freely among spawning media. Weak descriptions of territorial behaviors were described by Carranza and Winn (1954) in *F. notatus*, but similar results were not observed in *F. olivaceus*. The weak territorial behaviors observed within the genus *Fundulus* could be due to the lack of nesting behavior or spawning substrate preference with the genus of *Fundulus* (Blanchard 1998, Carranza and Winn 1954). With unlimited access to spawning substrates, *F. olivaceus* males are more likely to roam or follow behind females and mate anytime the female becomes receptive to the male (Carranza and Winn 1954). Another reason for the lack of territorial behavior may also be due to the spatial dynamics of the tank in which the tank size limits males to a smaller territory size that is found in natural conditions.

For the third hypothesis, I rejected the null hypothesis and found that a dominant male is established among spawning groups in *F. olivaceus*. With the use of genetic data, I was able to determine that sneaking behavior does not occur, and I assumed that all eggs found in the tank over the course of a mating event were fertilized by one male. These results suggest that a hierarchal system among the males exist in which one male is dominant over other males. Such evidence supports previous studies that an alpha male



interrupts any attempts by a subordinate male to mate with a female, and the alpha male is the only male to mate with the females present (Carranza and Winn 1954).

In the spawning groups in which males of the same phenotype were placed together, only one male mated with the female supporting that female *F. olivaceus* are selecting for a male and are not being forced to mate with the most aggressive male present. In previous studies within the genus *Fundulus*, limited male parental investment has been document (Carranza and Winn 1954) and suggests that since the female parental investment exceeds the male's investment, the reproductive success of the male is determined by the selection of the female (Kodric-Brown 1977; Trivers 1972). In a previous study, the results showed that female *Fundulus olivaceus* were highly selective when given a choice of a male of the same species and a male of another species (Jablonski 2009). For *F. olivaceus*, the females always selected the male of the same species (Jablonski 2009).

At the same time, male dominance does appear to have a factor in which male the female mates. In similar situations when a female is presented with two males of similar phenotype, the female is unable to distinguish between males and mates equal among the two (Draud and Itzkowitz 2004). The results I collected imply that the lack of promiscuity seen in females is related to another factor, male dominance.

#### Uncontrolled Variable

Although the study concentrated on secondary sex characteristics, size and spots, other factors, such as and intrasexual competition among males, may be a factor in reproductive behaviors observed in *F. olivaceus*.

Because females were collected throughout the breeding season, there was variation in female size and health in each trial. Although large bellied females (those appeared to have eggs) were selected, size or overall condition was not controlled. Since females were collected from the wild, previous exposure and preferred characteristics to sexually mature males is unknown.

#### Potential Bias

The size of the tank limited the mobility of the males and female(s) present and may have induced behaviors not observed in the wild. The limited space may have increased the chances of a female mating with an unattractive male due to the female's unwillingness to reabsorbing energetically costly eggs. The lack of spawning behavior in 10 trials in which no eggs were collected could also be due to female condition and not the female selecting against the present males.

Another bias could have occurred due to the female's inability to swim away from the male in search of another mate. In *Cyprinodon variegatus*, Draud and Itzkowitz (2004) found that if a higher quality male pupfish enters an area where a female is mating with a male she will leave the current male and mate with the new, higher quality male. As for *Fundulus olivaceus*, the lack of promiscuous behavior of males and females in the spawning tank may be the result of the alpha males being capable of observing any attempt of other males to mate with the female that may not be observable in the wild. If the male was able to visualize any attempt of another male to mate with the female, he could have prevented fertilization from occurring.



### Future Direction

It is unclear whether or not the nonrandom mating is strictly intrasexual selection between males, female mate selection, or a combination of both. Further research should concentrate on social interactions between males and females and whether an indirect or direct gain is acquired when a female mates with an alpha male. In tilapia (Cichlidae), *Oreochromis mossambicus*, social interactions between males influenced the hierarchal male system and the levels of androgens circulating within the plasma (Oliveira and Almada 1998). Androgens, which regulate secondary sex characteristics, as well as competitive and mating behaviors, are found in higher concentrations in dominant males than satellite or helper males (Oliveira and Almada 1998). At the same time, a high level of stress hormones suppresses the development of testes and reduces a male's reproductive fitness (Oliveira and Almada 1998). A similar approach should be considered when assessing dominance in male *F. olivaceus* due to the lack of sneaking behavior seen in subordinate males.

### Conclusion

Overall the study addressed reproductive behaviors in the blackspotted topminnow, *Fundulus olivaceus*, finding that within a group of breeding males, females do not mate randomly and select for a secondary sex characteristics of large body size and a high density of dorsolateral spots. As for male *F. olivaceus*, males will spawn randomly in unguarded spawning media and not guard a limited territory making one male the dominant male that sires all offspring.

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